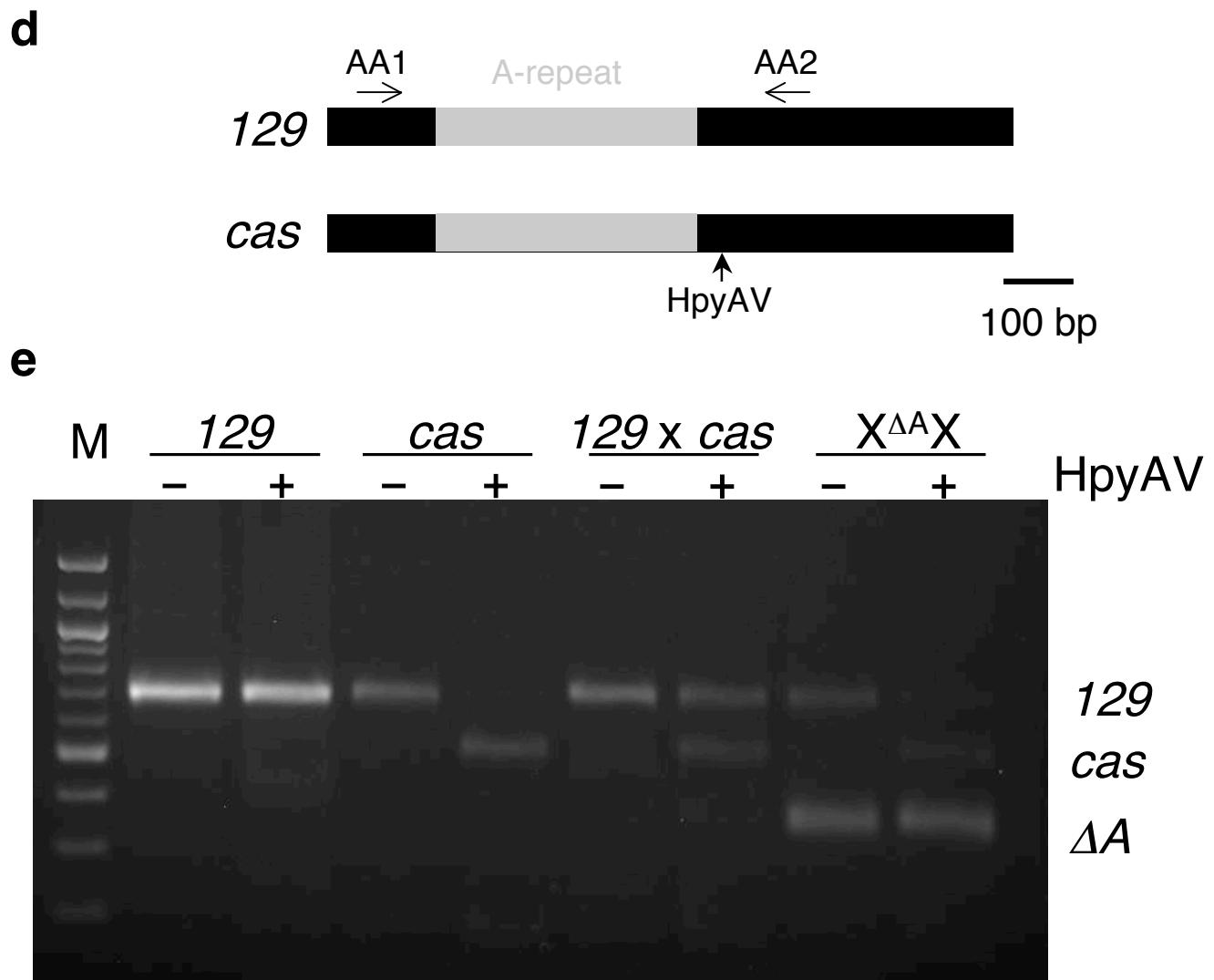
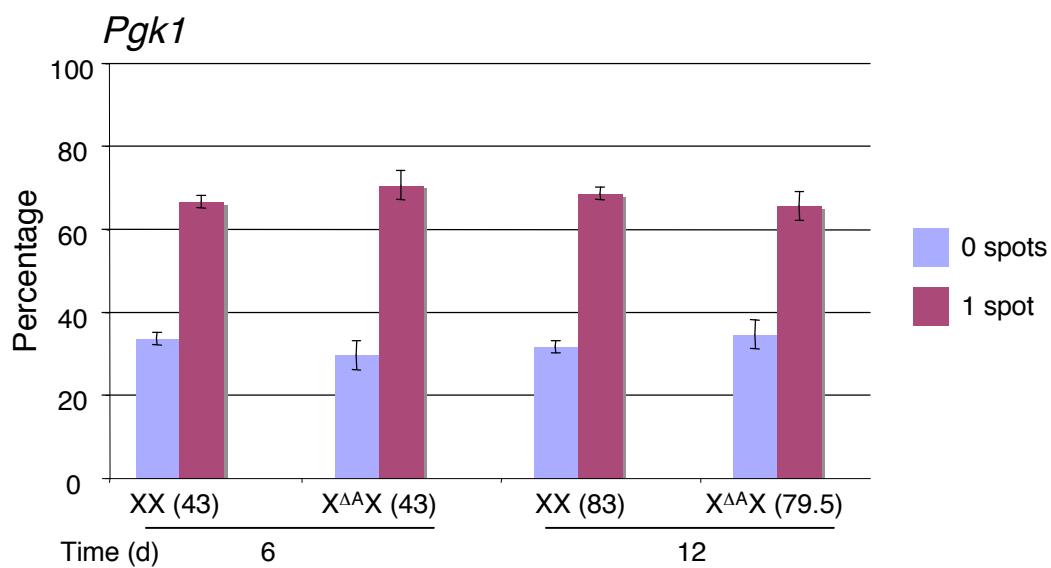
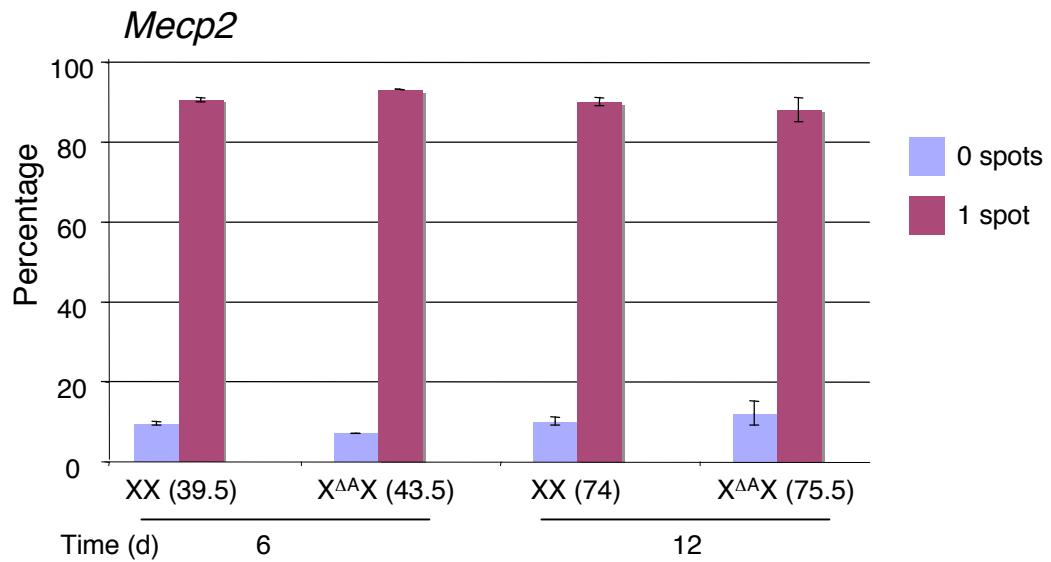


Supplementary Fig. 1. Characterization of $X^{\Delta A}X$ ES cells. (a) Targeting scheme for generating the $X^{\Delta A}$ allele. Positions of the relevant restriction enzyme sites and probes used in (b) and (c) are indicated. Triangles represent loxP sites. H, HindIII; D, DraI. (b) Homologous recombination in XX ES cells confirmed by Southern blot hybridization. Probes and restriction enzymes used are shown below and to the left of the blot, respectively. Correct band sizes are indicated on the right. (c) Excision of the puromycin-resistance cassette by Cre-recombinase was confirmed by Southern blot hybridization. Probes and restriction enzymes used are shown below and to the left of the blot, respectively. Correct band sizes are indicated on the right.



Supplementary Fig. 1. (cont'd) **(d)** Map indicating name and location of primers and location of restriction enzyme polymorphism, used to assess whether the *129* or *cas* allele was targeted. **(e)** Allele-specific analysis of the A-repeat region, demonstrating that the A-repeat is deleted from the *129* X in $X^{\Delta A}X$ ES cells.

f**g**

Supplementary Fig. 1. (cont'd) (f,g) Graphs showing the percentage of cells with Xist RNA coating (Percentage) that exhibited 0 (blue bars) or 1 (purple bars) sites of transcription for (f) *Pgk1* or (g) *Mecp2* RNA in XX or X^{ΔA}X ES cells at day (d) 6 or 12 of differentiation. The numbers in brackets beside the names of the cell lines indicate the average percentage of cells in the population with Xist RNA coating. In all cases >200 cells were counted from more than two biological replicates. Cells with Xist RNA coating that exhibited 2 sites of transcription for either gene were <1%, and are not included on the graph. Error bars indicate one standard deviation.

hPercentage singlet

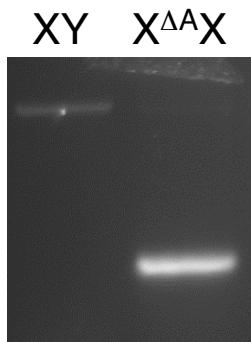
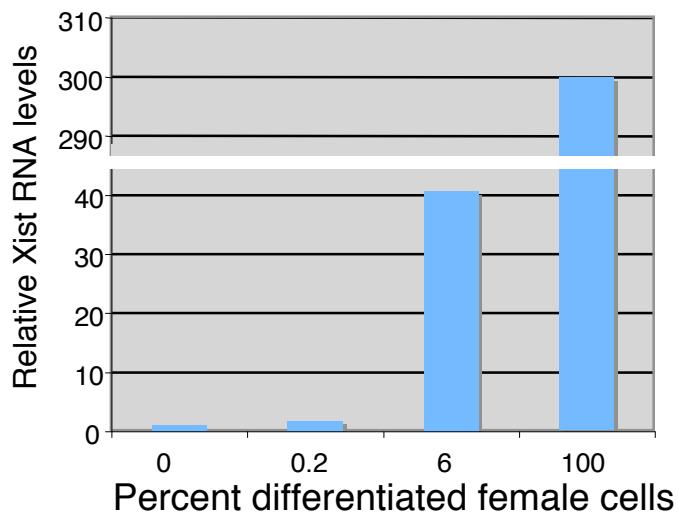
Cell line	Locus	WT	Mutant	(n)	P
$X^{TpA}X$	<i>Xist/Tsix</i>	30	70	120	<0.01
	<i>Pgk1</i>	68	32	125	<0.01
$X^{\Delta Xist}X$	<i>Xist/Tsix</i>	69	31	135	<0.01
	<i>Pgk1</i>	33	67	122	<0.01
$X^{\Delta A}X$	<i>Xist/Tsix</i>	69	31	128	<0.01
	<i>Pgk1</i>	34	66	113	<0.01

Supplementary Fig. 1. (cont'd) (h) The proportion of SD cells in which the wild-type (WT) or mutant X exhibited a singlet FISH signal for *Xist/Tsix* or *Pgk1* RNA in the ES cell lines indicated (Percentage singlet). Both *Xist/Tsix* and *Pgk1* RNA exhibited the same high frequency of SD cells in XX, $X^{TpA}X$ and $X^{\Delta Xist}X$ as previously reported using DNA FISH (data not shown). $X^{\Delta A}X$ ES cells exhibit the same high frequency of SD FISH signals for X-linked genes as wild-type, and heterozygous *Xist* or *Tsix* mutant female ES cells (data not shown), indicating that deletion of the A-repeat did not disrupt the overall frequency of cells with SD FISH signals. *Tsix* and *Xist* mutant ES cells exhibit primary X-inactivation and the future Xa is in orange and the future Xi is indicated in purple. *Xist* shows a high frequency of singlet FISH signals on the future Xi and other X-linked loci, like *Pgk1*, show a low frequency. The opposite pattern is observed on the future Xa. $X^{\Delta A}X$ ES cells exhibit the same pattern of singlet FISH signals at *Xist* and *Pgk1* as the *Xist* mutant line. (n) indicates the number of nuclei scored, P values indicate that the observed frequency of singlet FISH signals is significantly different from that in cells with random X-inactivation.

a

TGTTTGCTCG TTTCCCGTGG ATGTGCGGTT CTTCCGTGGT TTCTCTCCAT **CTAAGGAGCT**
TTGGGGGAAC ATTTTAGTT CCCCTACCAC CAAGCCTAT GGCTTATTAA AGAAAACATA
 TCAAAATTCC ACGAGATT TT TGACGTTTG ATATGTTCTG GTAAGATTT TTTTTGACA
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 TCCTTAGCCC ATCGGGGCCA TGGATACCTG CTTTTGTAA **AAAAAAAAAA** **AAAAAAAAAA**
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 ACGGGGCCTC GGATACCTGC TGTTATTATT TTTTTTCTT TTTCTTTGC CCATCGGGC
 TGTGGATACC TGCTTAAAT TTTTTTTA CAGGCCAAC GGGGCTTGGT GGATGGAAAT
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CGGGATTTCGC CTTGATTTGT **GGTAGCATTT** GCAGGGTTGT GCTAGCCGGA AGAGAAAGCC
 AAGGAGTGCT CGTATTAGTG TGCAGTGTG CGCGGAAGCC GCAGAGGACT AGGGGATAGG
 GCTCAGCGTG GGTGTGGGG

Supplementary Fig. 2. Characterization of in X^{AA}Y ES cells. (a) The sequence of the A-repeat region in X^{AA}Y ES cells, which are derived from a 129 strain background. Red text indicates the A-repeat, purple text indicates the region deleted in X^{AA}Y ES cells, and green text indicates the PCR primers used for genotyping in (b) (listed in **Supplementary Table 1**).

b**c**

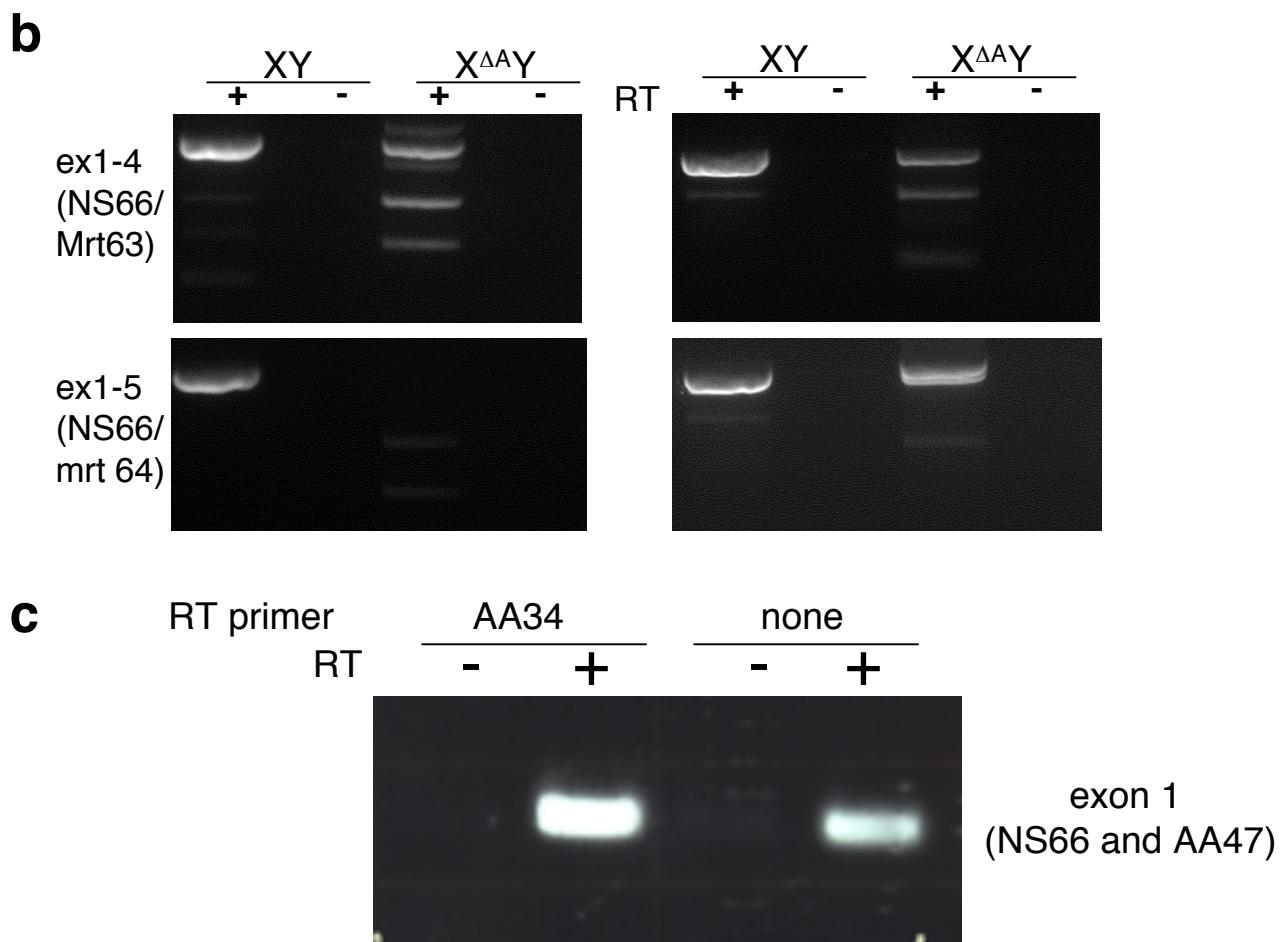
Supplementary Fig. 2. (cont'd) **(b)** PCR genotyping of $X^{\Delta A}Y$ ES cells and parental XY cells.

(c) Even when feeders and differentiated cells were depleted by preplating on gelatinized dishes, we routinely observed ~0.5% of cells with Xist RNA coating (coaters) in undifferentiated XX and $X^{\Delta A}X$ female but not male ES cells. These coaters likely represent differentiating female cells undergoing X-inactivation, as it is well known that there is some differentiation in ES cell cultures even when they are grown under conditions that promote self-renewal. Because steady state levels of Xist RNA increase dramatically upon differentiation, we were concerned that differentiated cells could skew the *129* to *cas* ratios in our allele-specific RT-PCR assays. To test whether a small proportion of coaters could significantly impact Xist RNA levels in undifferentiated ES cell populations, we determined the amount of spliced Xist RNA in male ES cells that were spiked with 0%, 0.2%, 6%, or 100% of differentiated female cells ($X^{\Delta A}X$ female ES cells, day 8 of differentiation). All samples were normalized to the male ES cell sample, which was set to 1. The addition of 0.2% of differentiated female cells resulted in a nearly 2-fold increase in steady state levels of Xist RNA, indicating that even the small proportion of coaters in undifferentiated female ES cells has the potential to skew *129:cas* ratios.

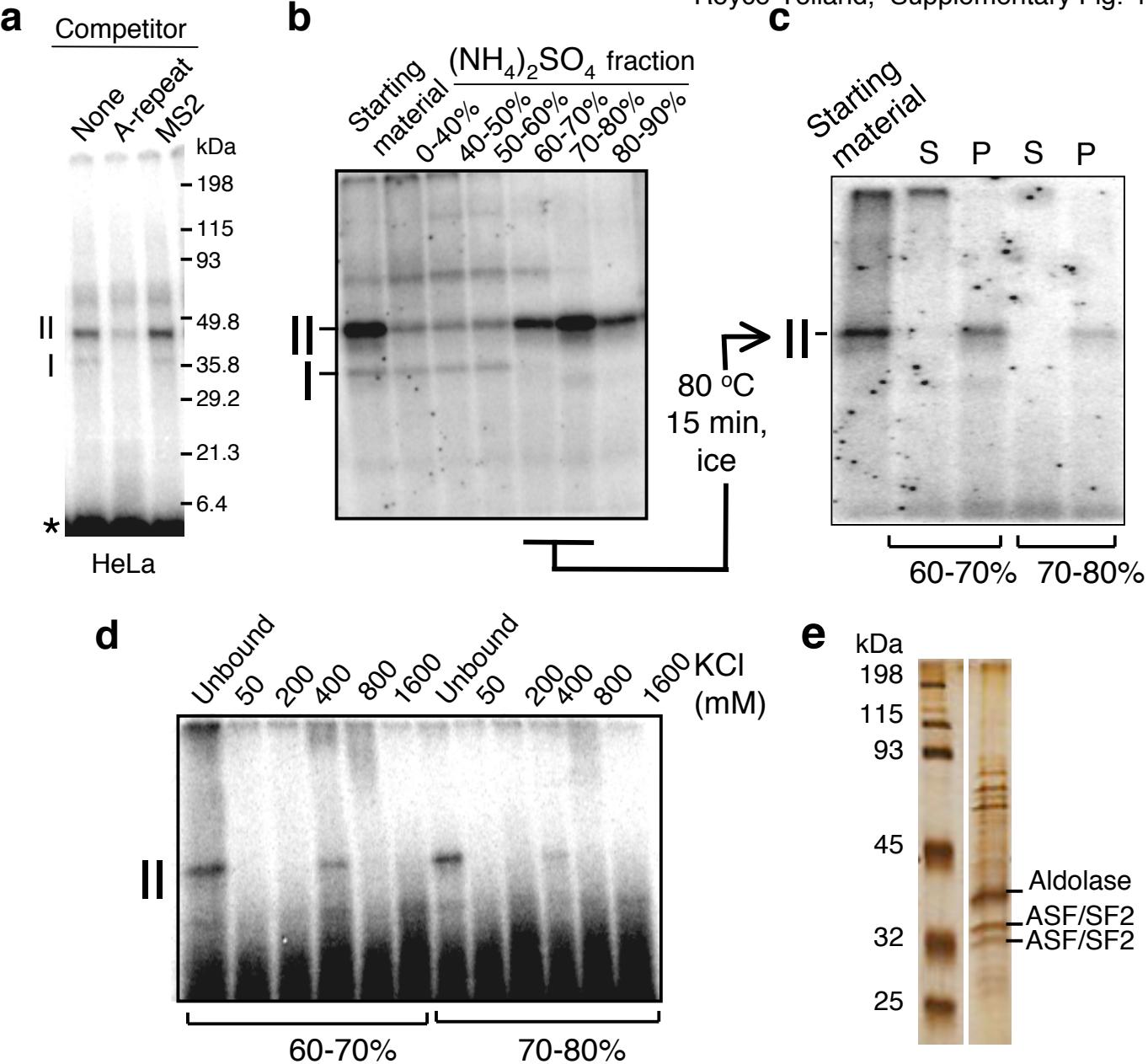
a

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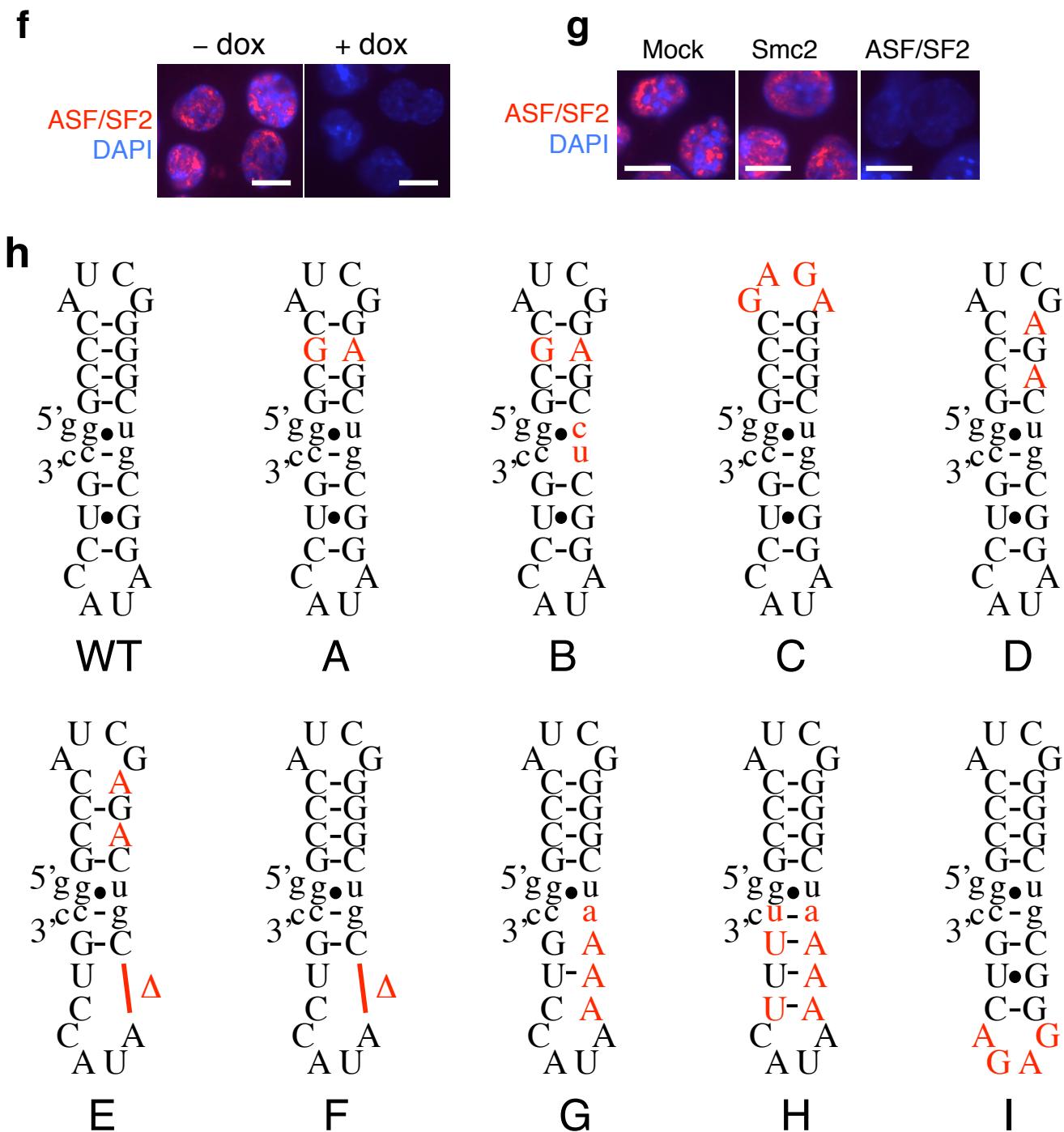
Supplementary Fig. 3. Spliced Xist RNA analysis in X^{ΔA}Y ES cells. (a) Sequence of the ΔA exon 3-6 RT-PCR product from X^{ΔA}Y ES cells, in red. *Xist* exons are in upper case and intronic sequences are lower case. Black sequences are absent in the ΔA RT-PCR product.



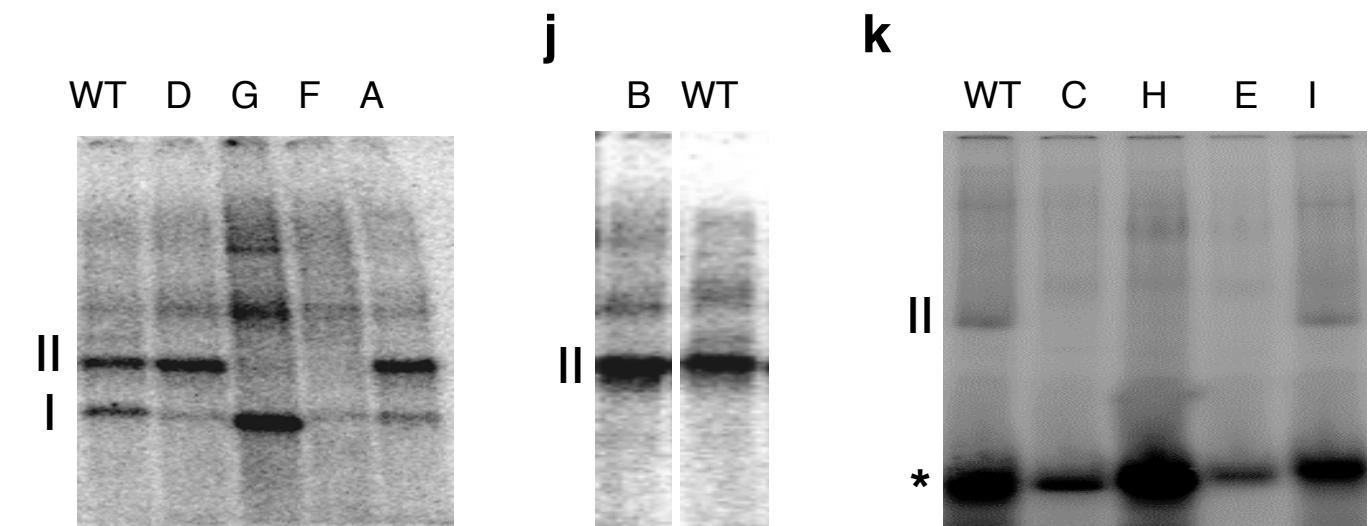
Supplementary Fig. 3. (cont'd) (b) RT-PCR products produced using random primed cDNA and PCR primers within exons indicated on the left. Primer names indicated in brackets beneath exons. Two examples are given to show the variability in the aberrant products produced in male Δ A ES cells. None of these aberrant products are the sizes expected from Tsix RNA or genomic DNA. All primer sequences are listed in **Supplementary Table 1**. (c) RNA from male ES cells was reversed transcribed with and without primers. At high frequency, RT-PCR products could be detected when there was no primer in the reverse transcriptase reaction. This indicates that Xist and Tsix RNA can prime each other, as has been demonstrated for other antisense RNA pairs.



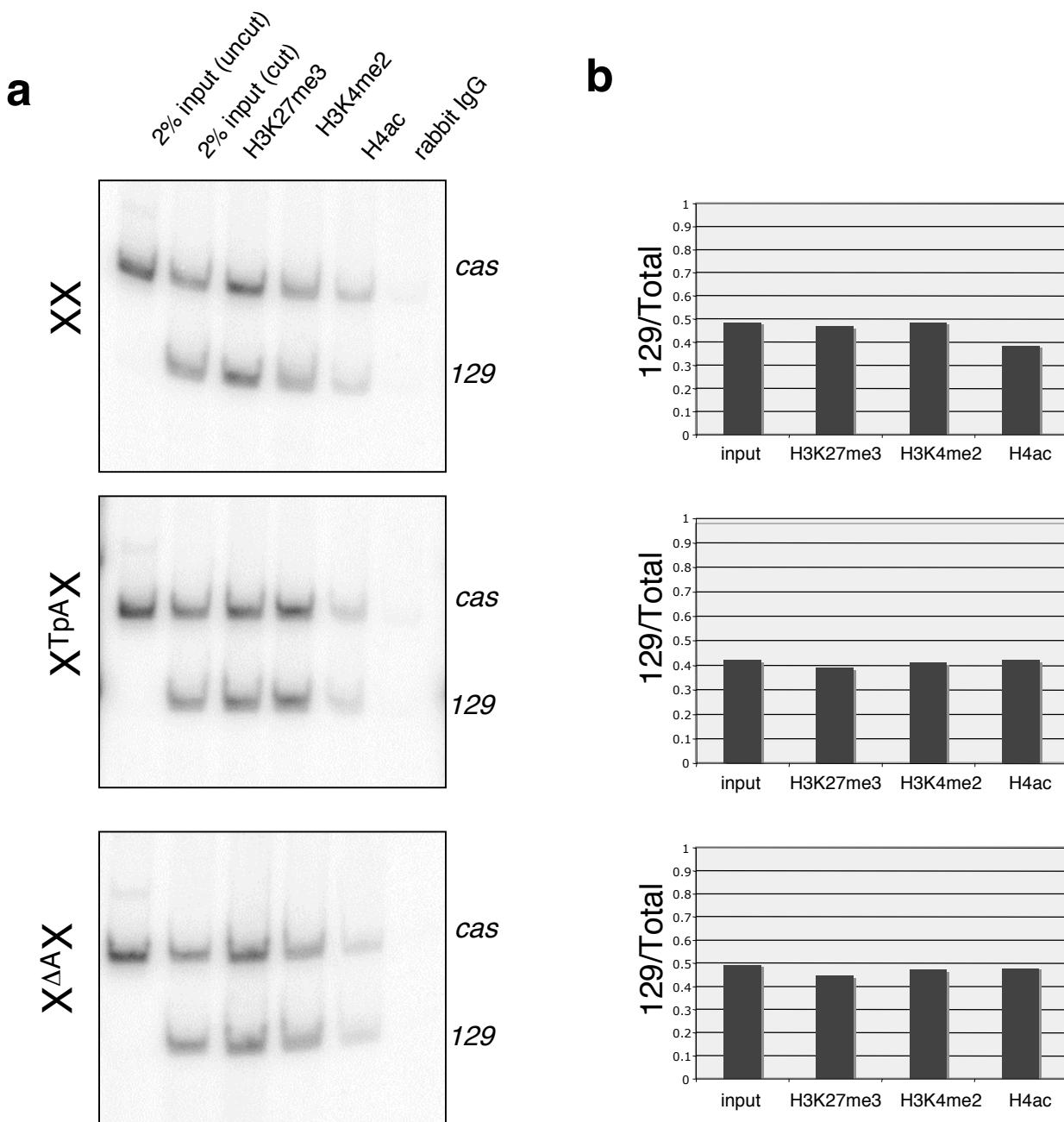
Supplementary Fig. 4. Identification of ASF/SF2. (a) SDS-PAGE gel of material UV cross-linked with radio-labeled A-repeat RNA. Sizes of molecular weight markers are indicated on the right. These results indicate that the A-repeat binds proteins in the 30 to 40 kDa range. (b-d) Partial purification of A-repeat binding activities. In all instances, A-repeat binding activities were followed using gel mobility shift assays. (b) HeLa cell nuclear extracts were fractionated using $(\text{NH}_4)_2\text{SO}_4$ at percentages indicated. (c) 60-70% and 70-80% $(\text{NH}_4)_2\text{SO}_4$ fractions were each incubated at 80°C for 15 minutes, cooled on ice, and pelleted. Supernatant (S). Pellet (P). (d) Supernatants from (c) were subject to Q-sepharose batch affinity chromatography, and material eluted with KCl at increasing concentration. Binding activity was enriched approximately 50-fold in the flow through. (e) Silver stained SDS-PAGE gel of partially purified extract in which the A-repeat binding activity comprising complex II was purified. Molecular weight markers are indicated on the right. The most prominent bands in the 30-40 kDa range (indicated) were excised and identified by mass spectrometry. The two bands indicated both contained ASF/SF2, which migrates as a dimer because a fraction is phosphorylated.



Supplementary Fig. 4. (cont'd). **(f)** Immunostaining for ASF/SF2 (red), which is used to show the efficiency of depletion in Fig. 4e. Nuclei are stained with DAPI (blue) and scale bars are 5 microns. **(g)** Immunostaining images show ASF/SF2 levels (red) in mock, Smc2 and ASF/SF2 knockdown ES cells (Fig. 4f). Nuclei are stained with DAPI (blue) and the scale bar indicates 5 microns. **(h-k)** Mutational analysis of the A-repeat subunit consensus sequence. **(h)** A-repeat RNA sequences assayed, with mutations indicated in red.



Supplementary Fig. 4. (cont'd). **(i, j)** UV cross linking with mutant RNAs indicated and controls. **(k)** Gel mobility shift of mutant RNAs indicated and controls. * indicates unbound RNA. Complex I and/or II are indicated. Complexes I and II are formed from HeLa nuclear extracts, and only complex II is formed using partially purified HeLa extracts.



Supplementary Fig. 5. ChIP analysis at the *Xic* hotspot. The H3K27me3 hotspot, which lies 50kb upstream of the *Xist* promoter, exhibits roughly equal distributions of modified histones between the 129 and *cas* chromosomes in XX, X^{TpA}X, and X^{AA}X ES cells. **(a)** Allele-specific ChIP for H3K27me3, H3K4me2, and H4ac in XX, X^{TpA}X, and X^{AA}X ES cells. Primers and restriction enzyme are indicated in **Supplementary Table 1**. **(b)** Data from (a) plotted as the proportion of signal from the 129 allele.

Supplemental Table 1. Primer sequences.

assay	amplicon	name	sequence	enzyme
Allele-specific ChIP	Xist promoter	XP-3F *X1-17R	CGTCATGTCAGTGAGCTTAC GAGAAACCACGGAAAGAACCG	Tsp509I
	Xist exon 7	*X7-7F+2	TCTTTCTGTTCACTTTGAGC	MnII
	Tsix promoter	X7-10R *E-f E-r	GGCGTTCACTTCAGAGCCACTTG CTTCAAACCTGCAAAGCTCT	
	Hotspot	*HotspotP-6f HotspotP-6r	GTCTGCCTACTAACACAGGT GGAAAGGTTCTCCATCTT	Asel
			GGAAAACCACATCTGGTTGA	MseI
RT-PCR	Xist (ex1-3)	NS66 NS33	GCTGGTTCGTCTATCTTGTGGG CAGAGTAGCGAGGGACTTGAAGAG	ScrFI
	Xist (ex3-6)	mx23 mx20	ATGTTGATCCTCGGGTCATTAT ACTGCCAGCAGCCTATAACAG	
	Tsix (ex3-4)	21580F AA51	21580F CTTGCAGACGCTACACACTT	ScrFI
	β-actin	β-actin F β-actin R	GGCCCAGAGCAAGAGAGATCC ACGCACGATTTCCTCTCAGC	
	Xist (ex1-4)	NS66 mrn63	GCTGGTTCGTCTATCTTGTGGG CCCAGTGGTGGTGAGCTATT	
	Xist (ex 1-5)	NS66 mrn64	GCTGGTTCGTCTATCTTGTGGG AGAATGGCTTCCTCGAAGGT	
	strand-specific RT-PCR/RT-qPCR	*T3 mrt45 mrt46/T3	AATTAACCCCTCACTAA CTTGCAGGGGATACCGTTA AATTAACCCCTCACTAAAGGGGTTCAAGTGCACAGAGCAGGT	
	Xist (ex1-in1)	NS66 AA34/T3	GCTGGTTCGTCTATCTTGTGGG AATTAACCCCTCACTAAAGGGGTTGGGTGGCACAGAAAGAAACTCGAATG	ScrFI
RNA IP	XIST wild type	WTf WTr	GTGGATCCAGTTTTACTCTTCCA ACCCACAAAACCAACATTTTCATC	
	XIST A-repeat deletion	DeltaAf DeltaAr	GTCCCCAACACCCCTTATGGCGT CCAACAGAGTAAAAAACTGATCCAC	
	Southern	probe 1 probe 2	DP2a DP2b	CATGGGTGCTATGCCCGAGTCAC AAGGCTAGCCTGGGTTATATGCTAA TATTAATAGTAAATCAATTAC CGGGCCGAGATTATAAAC
	No primer RT	Xist (ex1-in1)	AA47 AA34 NS66	ATGAGAAAAAGATAGCTAG GTGGGTGGCACAGAAAGAACTCGAATG GCTGGTTCGTCTATCTTGTGGG
	Cloning	5' targeting arm 3' targeting arm	5'HR-forward 5'HR-reverse 3'HR-forward 3'HR-reverse	AACTTTATGTGAATGTCATTAG AGATTATAAAACAATGAAAGAAAGG GTGGATGGAAATATGGTTGTGAG AGCAATAGCAGCAGCACTATTG
	qPCR	Tsix	mrt104 mrt105	CCAAGCAGCAGAAAGATTCC AAGGACGTGAGTTCGCTTG
	β-actin	β-actin F β-actin R	GGCCCAGAGCAAGAGAGGTATCC ACGCACGATTTCCTCTCAGC	
	Xist (ex3-6)	mx23 mx20	ATGTTGATCCTCGGGTCATTAT ACTGCCAGCAGCCTATAACAG	
	Xist (ex1-in1)	NS66 AA34	GCTGGTTCGTCTATCTTGTGGG GTGGGTGGCACAGAAAGAACTCGAATG	
FISH probes	Xist ex1-pooled	ex1-5a ex1-5b ex1-6a ex1-6b ex1-7a ex1-7b ex1-8a ex1-8b ex1-9a ex1-9b ex1-10a ex1-10b	GTCCATGGACAAGTAAACAAAAGAAC TATGAGGGTATGGATCTGGTTA GATCCCATACCCCTCATACCCCTAAT CTTGAAGGACCATGGACCGTATT TGCCTTATGGAATTATGTATGTG GGTCCGAAAAGTAATAAGGTTGTG ACTTTCGGACCATGGTATCTT GAGAGCAGGTATTGTCAGAG TCCCCTGCTAGTTCCCAATGT TTTCCACAGACTCATACCCCTCAG TTTTAAAAGGTGACTGGATGGTT TGATGTAACCGAGGAGCAGTAG	
	Tsix probe	X3'1F X3'1R	AACCACTGCCACATCCCCCTTTC CCCTCCCGCCCTGGCCAGCACCCCT	
	genotype XΔAY	mrt111 mrt112	CTAAGGAGCTTGGGGGAAC	
	genotype XΔAX	AA1 AA2	ACCACAAATCAAGGCAGAAC GACATGCTCCCATACTTTG CATAGGTTCACTCACACAG	
	Smc2 esiRNA	Smc2f Smc2r	GGGCGGGTGCCTTCAAGTTCCAATCTTCA GGGCGGGTCAAATACATCACCCCCCAAGG	
	ASF/SF2 esiRNA	Asff Asfr	GGGCGGGTCTGTGAGGCAGGTGATGT GGGCGGGTCCAAGACATGAGGGGAATG	
		T7 adaptor	TAATACGACTCACTATAGGGAGACCAACGGCGGGT	